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Oncostatin M-stimulated Apical Plasma Membrane Biogenesis Requires p27^{Kip1}-Regulated Cell Cycle Dynamics

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Oncostatin M regulates membrane traffic and stimulates apicalization of the cell surface in hepatoma cells in a protein kinase A-dependent manner. Here, we show that oncostatin M enhances the expression of the cyclin-dependent kinase (cdk)2 inhibitor p27^{Kip1}, which inhibits G₁-S phase progression. Forced G₁-S-phase transition effectively renders presynchronized cells insensitive to the apicalization-stimulating effect of oncostatin M. G₁-S-phase transition prevents oncostatin M-mediated recruitment of protein kinase A to the centrosomal region and precludes the oncostatin M-mediated activation of a protein kinase A-dependent transport route to the apical surface, which exits the subapical compartment (SAC). This transport route has previously been shown to be crucial for apical plasma membrane biogenesis. Together, our data indicate that oncostatin M-stimulated apicalization of the cell surface is critically dependent on the ability of oncostatin M to control p27^{Kip1}/cdk2-mediated G₁-S-phase progression and suggest that the regulation of apical plasma membrane-directed traffic from SAC is coupled to centrosome-associated signaling pathways.

INTRODUCTION

The establishment and maintenance of apical and basolateral plasma membrane (PM) domains in epithelia require a careful orchestration of extra- and intracellular signals, triggering crucial cytoskeleton-, organelle-, and membrane traffic-linked machineries, as partly elucidated in systems as diverse as yeast, *Drosophila*, and mammalian cells (Lecuit and Pilot, 2003; Mostov *et al.*, 2003; Baas *et al.*, 2004). Impairment of these events may cause a loss of apical-basolateral asymmetry and may lead to deranged cell growth (Bilder *et al.*, 2000; Vermeer *et al.*, 2003; Baas *et al.*, 2004) or spindle misorientation (Albertson and Doe, 2003). Recent studies have reported an evolutionary conserved genetic program that may link the process of polarity development and proliferation (Bilder *et al.*, 2000; Humbert *et al.*, 2003; Rolls *et al.*, 2003; Klezovitch *et al.*, 2004). However, many aspects of such a link remain unclear, e.g., the extent to which cell proliferation can directly affect epithelial cell organization or vice versa. Indeed, epithelial cell proliferation and apical-basolateral polarity may be controlled by different signaling pathways (Liu *et al.*, 2004).

Signaling cascades stimulated by interleukin-6 family cytokines, including oncostatin M (OSM), elicit multiple responses in the cell. Originally, as its name indicates, oncosta-

tin M was primarily recognized to inhibit the proliferation of tumor cells (Tanaka and Miyajima, 2003), presumably by modulating cell cycle regulatory proteins, including the cyclin-dependent kinase (cdk)2-inhibitor p27^{Kip1} (Kortylewski *et al.*, 1999; Klausen *et al.*, 2000; Li *et al.*, 2001). Disruption of the regulatory system that controls G₁-phase progression is a common event in human hepatocarcinogenesis (Hui *et al.*, 1998), and p27^{Kip1} has been associated with differentiation, invasiveness, and metastasis of hepatocellular carcinoma tumors (Zhou *et al.*, 2003). Recent evidence indicate that OSM signaling plays a prominent role in liver development (Kamiya *et al.*, 1999, 2001, 2002; Kojima *et al.*, 2000; Kinoshita and Miyajima, 2002; Matsui *et al.*, 2002; Chagraoui *et al.*, 2003; Hanada *et al.*, 2003; Lazaro *et al.*, 2003). OSM stimulates hepatocyte nuclear factor-4 (Kamiya *et al.*, 2003), which may integrate the genetic programs of liver-specific gene expression and epithelial morphogenesis (Spath and Weiss, 1998). In addition, OSM induces adherens junction formation by altering the subcellular localization of adherens junction components, including E-cadherin and catenins (Matsui *et al.*, 2002). In human fetal hepatocytes as well as in well-differentiated human hepatic HepG2 cells, OSM stimulates the development of apical bile canalicular plasma membrane (PM) domains (Lazaro *et al.*, 2003; van der Wouden *et al.*, 2002), which requires the activation of a gp130-mediated signaling cascade and endogenous protein kinase A (PKA) activity (van der Wouden *et al.*, 2002). Although the molecular mechanisms that underlie apicalization of the cell surface in hepatocytes are poorly understood, the de novo reorganization of the lateral PM after the establishment of E-cadherin-dependent cell-cell adhesion, mediated by cytoskeleton rearrangements and the polarized sorting and trafficking of proteins and lipids, plays a crucial role.

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Apical protein and lipid sorting in polarized hepatocytes occurs in the Golgi apparatus and the subapical compartment (SAC; the hepatocyte equivalent of the pericentrosomal recycling endosomes in other epithelia). In the Golgi apparatus, newly synthesized proteins and lipids destined for the apical surface are sorted into sphingolipid-cholesterol-enriched membrane microdomains (rafts). Thus, in the *trans*-Golgi network, multimembrane-spanning or polytopic apical membrane proteins are clustered in Lubrol WX-resistant membrane domains and delivered via vesicular transport directly to the apical surface (Ait Slimane *et al.*, 2003). Single membrane-spanning apical proteins are segregated in other, Triton X-100-resistant membrane domains and targeted first to the basolateral surface, followed by their transport to the apical domain by transcytosis (Ait Slimane *et al.*, 2003; Nyasae *et al.*, 2003; Tuma and Hubbard, 2003). After surface delivery extensive membrane exchange occurs between the apical and basolateral domains, which involves endocytosis and subsequent recycling to either PM domain. The SAC plays a prominent role in the polarized delivery of proteins and lipids, and thus in maintaining cell surface polarity (Ihrke *et al.*, 1998; van IJzendoorn and Hoekstra, 1999a,b; Rahner *et al.*, 2000; van IJzendoorn and Mostov, 2000). Moreover, polarized membrane traffic from SAC is also important for the *de novo* biogenesis of apical PM domains (van IJzendoorn and Hoekstra, 2000), triggered by extracellular stimuli, including OSM (van der Wouden *et al.*, 2002) and those that elicit cAMP/PKA signaling cascades (Zegers and Hoekstra, 1997). Indeed, we have previously shown that OSM activates a vesicular membrane trafficking pathway from SAC to the apical PM, which is dependent on cAMP/PKA activity (van der Wouden *et al.*, 2002). Importantly, activation of this OSM-cAMP/PKA-regulated pathway is crucial for apical PM biogenesis in HepG2 cells (van der Wouden *et al.*, 2002).

The pronounced effects of OSM on cell cycle progression, membrane traffic, and cell polarity prompted us to investigate the functional relationship between these events to obtain better insight into molecular mechanisms that control the establishment of apical surface domains in conjunction with cell cycle control.

MATERIALS AND METHODS

Cell Culture

HepG2 cells were grown in DMEM with 4500 mg of glucose per liter, containing 10% fetal calf serum and antibiotics. Media were changed every other day. Cells were trypsinized after reaching confluence. For experiments, cells were plated at low density onto ethanol-sterilized coverslips and analyzed 24, 48, 72, or 76 h after plating.

To synchronize cells in the G₁ phase of the cell cycle, cells were first grown in normal culture medium for 36 h to allow expansion of the cells, followed by culture in serum-free medium for 36 h. In some experiments, cells were subsequently exposed to normal culture medium containing 10% fetal calf serum (FCS) or cultured in medium supplemented with 30 μ M fumonisin B1 (Sigma-Aldrich, St. Louis, MO; see *Results*), to chase the cells from G₁ into the S phase. As a control, cells were chased in serum-free medium. G₁-S-phase transition was monitored by the initiation of DNA synthesis, as evidenced by bromodeoxyuridine (BrdU) incorporation (Figures 2C and 3C). In other experiments, cells were first grown in normal culture medium for 36 h, followed by culturing in normal medium supplemented with the DNA synthesis inhibitor hydroxyurea (4 mM; Sigma-Aldrich) to synchronize the cells in S phase.

Determination of HepG2 Cell Polarity

Accurate estimation of the degree of HepG2 polarity was performed as described previously (Zegers and Hoekstra, 1997; van IJzendoorn and Hoekstra, 2000). Briefly, the cells were fixed with -20°C ethanol for 10 s, rehydrated in Hank's balanced salt solution (HBSS), and subsequently incubated with a mixture of tetramethylrhodamine B isothiocyanate-labeled phalloidin (Molecular Probes, Eugene, OR) and 5 ng/ml the nuclear stain Hoechst 33528

(Sigma-Aldrich) at room temperature for 30 min. The cells were then washed, and the number of bile canaliculi (BC; identified by the presence of dense F-actin staining around the BC) per 100 cells (identified by fluorescently labeled nuclei) was determined and expressed as the ratio [BC/100 cells]. Ten fields (each containing >50 cells) per coverslip (at least two coverslips per condition were studied) were analyzed. Cells were examined using an epifluorescence microscope (Provis AX70; Olympus, New Hyde Park, NY).

Immunostaining of HepG2 Cells

HepG2 cells were fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature, washed, and permeabilized for 10 min with methanol at -20°C . After blocking with 1% bovine serum albumin (BSA) in phosphate-buffered saline (37°C; 1 h) the cells were incubated with primary antibody for 1 h at 37°C. For immunofluorescent costaining of γ -tubulin and PKA, cells were first incubated with 0.5% Triton X-100 at 4°C for 3 min, followed by fixation with ice-cold (-20°C) methanol for 5 min and blocking with 1% BSA. Monoclonal anti-p27Kip1 and anti-PKA-R1 α antibodies were bought from BD Transduction Laboratories (Lexington, KY) and used at 1:300 dilution. After incubation with the primary antibodies, cells were washed and incubated with the corresponding secondary antibody labeled with Alexa Fluor⁴⁸⁸ or Alexa Fluor⁵⁹⁴ (Molecular Probes) for 30 min at room temperature. The coverslips were mounted and analyzed by confocal microscopy (TCS SP2; Leica, Wetzlar, Germany).

BrdU Incorporation Assay

HepG2 cells were incubated with 50 mM BrdU in normal culture medium (see *Results*). Cells were then washed with HBBS and fixed with 4% PFA at room temperature for 30 min. Cells were subsequently washed with HBBS and incubated in 2 N HCl for 1 h. Cells were processed for immunofluorescence microscopy as described above. In brief, cells were incubated at 37°C for 30 min with a mouse monoclonal antibody against BrdU (1:50 dilution; Chemicon International, Temecula, CA). As secondary antibody, Alexa⁴⁸⁸ goat anti-mouse IgG was used (Molecular Probes). During the secondary antibody incubation, 5 ng/ml nuclear stain Hoechst 33528 was included to visualize the nuclei. The cells were then rinsed four times with HBBS, mounted, and examined by fluorescence microscopy as described above. The percentage of cells that had incorporated BrdU was quantified by determining the ratio of BrdU-positive nuclei. At least 10 fields each containing >50 cells were counted.

Analysis of p27^{Kip1} Levels by Western Blot

HepG2 cells were treated as described in *Results* and then lysed in 0.5 ml of Triton X-100 lysis buffer (10 mM triethanolamine, pH 7.4, 1.0% Triton X-100, 0.1% SDS, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₄P₂O₇, and 2 mM sodium vanadate and a cocktail of protease inhibitors). Lysates were boiled for 5 min and cleared by centrifugation. Protein concentrations were determined by a bicinchoninic acid protein assay (Sigma-Aldrich), and equal amounts of proteins were separated on 10% SDS-PAGE gels, immunoblotted with monoclonal p27^{Kip1} antibodies (BD Transduction Laboratories) and detected with an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). Bands representing p27^{Kip1} were quantified using Scion Image software.

Analysis of Transport of C₆-NBD-GalCer from SAC

To study the trafficking of the fluorescent GalCer analog from SAC, SAC were preloaded with the lipid as depicted in Figure 1. This procedure has been described in detail previously (van IJzendoorn and Hoekstra, 1999a, 2000). In short, cells were labeled with 4 μ M C₆-NBD-GalCer at 37°C (Figure 1, step 1) to allow internalization from the basolateral surface and subsequent transcytosis to the apical BC PM domain (Figure 1, step 2). After 30 min of incubation, lipid analog still residing at the basolateral domain was depleted by a back exchange procedure at 4°C [2 \times 30 min incubation in HBSS + 5% (wt/vol) BSA; Fluka, Fuchs, Switzerland; c.f. van IJzendoorn *et al.*, 1997] (Figure 1, step 3), and BC-associated lipid analog was chased into SAC at 18°C for 1 h in back exchange medium (Figure 1, step 4). Then, the NBD fluorescence at the exoplasmic BC leaflet was abolished using sodium dithionite (Merck Biosciences, Darmstadt, Germany) at 4°C, leaving the majority of the intracellular GalCer analog in SAC (Figure 1, step 5; van IJzendoorn and Hoekstra, 1998, 2000). After removal of dithionite by washing, transport from SAC was examined by incubating in back exchange medium at 37°C (Figure 1, step 6).

To quantitate transport of the lipid analog to and from the BC, the percentage of NBD-positive BC was determined as described previously (van IJzendoorn *et al.*, 1997; van IJzendoorn and Hoekstra, 1998). Briefly, BC were first identified by phase contrast illumination and then categorized as NBD-positive or NBD-negative under epifluorescence illumination. Note that a BC is categorized as fluorescently labeled, i.e., NBD-positive, when microvilli-like structures, characteristic of the BC, can be detected that are seemingly fluorescent in the wake of the fluorescence derived from the lipid analog, present in the apical membrane (van IJzendoorn *et al.*, 1997). Such microvilli-like structures are typically and readily observed upon gradual photobleaching of the BC-associated NBD fluorescence.

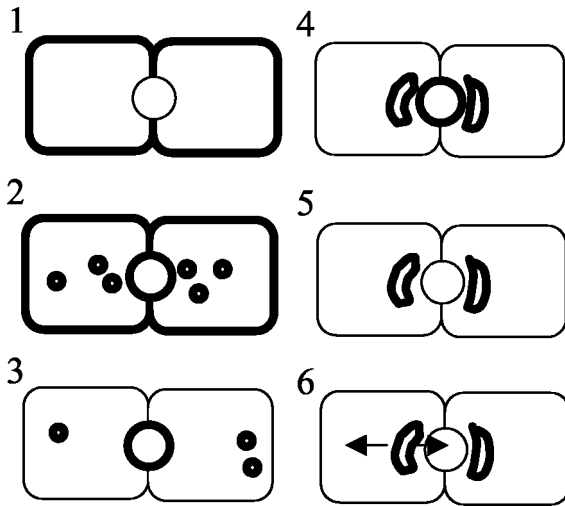


Figure 1. Schematic representation of the various steps of the lipid transport assays. The cell-labeling situation after the corresponding incubation step is depicted at the right. Cells are incubated with C_6 NBD-GalCer at 37°C for 30 min, which allows incorporation of the probe in the basolateral plasma membrane (step 1) and subsequent transcytosis to the apical domain (BC; step 2). Then, the fraction of the fluorescent lipid probe still residing at the basolateral PM is depleted by BSA at 4°C twice for 30 min (step 3). The BC-associated probe is then chased into SAC at 18°C for 1 h in BSA-containing medium (step 4). The presence of BSA in the medium during this step will prevent reentry of any C_6 NBD-GalCer that might arrive basolaterally during the chase. Next, the probe remaining at the BC is quenched by sodium dithionite (step 5). The sodium dithionite is removed by extensive washing with ice-cold HBSS. Occasionally, cells were subsequently incubated with OSM at 4°C for 30 min. Transport of the subapically derived fluorescent probe can be monitored upon rewarming and incubating the cells at 37°C (step 6).

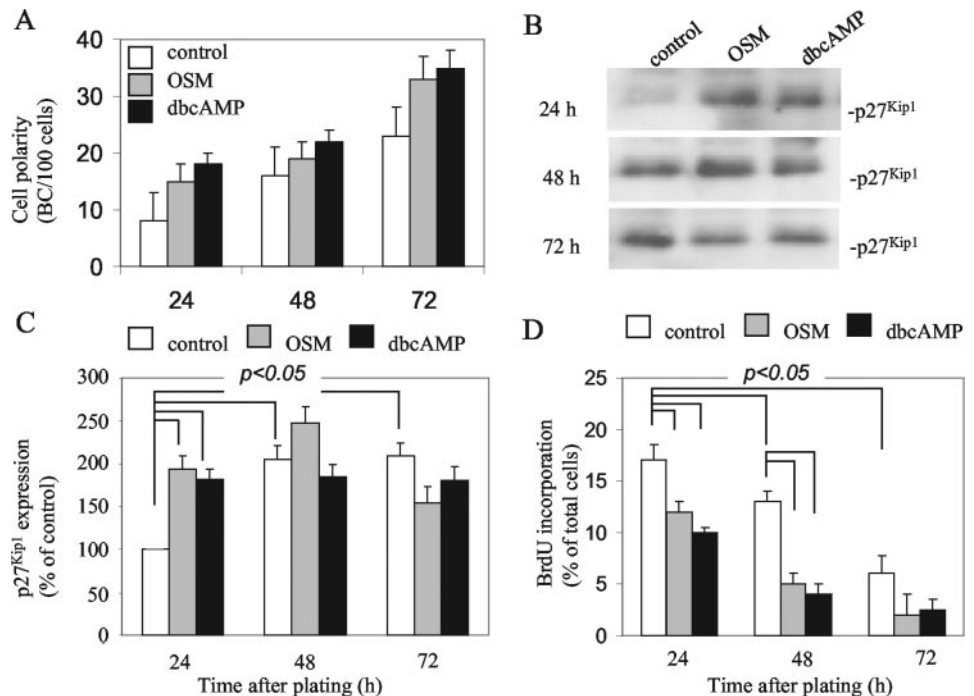
Distinct pools of fluorescence are thus discerned at the apical pole of the cells, present in vesicular structures adjacent to BC, which are defined as SACs (c.f. van IJzendoorn and Hoekstra, 1998). Together, BC and SAC constitute the bile canalicular pole (BCP) in HepG2 cells (Figure 1). Therefore, within the BCP region the localization of the fluorescent lipid analog will be defined as being derived from the BC, SAC, or both. This also provides a means to describe the movement of the lipid within or out of this region in the cell (van IJzendoorn and Hoekstra, 1999a). Thus, after loading SAC with lipid analog and allowing its transport to take place as described above, the direction of movement of the lipid from or within the BCP region is determined after a given time, by establishing the distribution of the NBD-labeled lipid over the various compartments (BC, SAC, or both) that constitute the BCP, relative to the labeling (i.e., primarily SAC), before starting the chase ($t = 0$). For this kind of analysis, at least 50 BCP per coverslip were analyzed. Data are expressed as the mean \pm SEM of at least four independent experiments, carried out in duplicate, and Student's t tests were carried out to determine the statistical significance of the data.

RESULTS

Cell Polarity Development Is Positively Correlated with $p27^{Kip1}$ Expression

Well-differentiated human hepatic HepG2 cells have been successfully used for the study of membrane dynamics and polarity development in hepatocytes. Apical BC PM domains form between two adjacent cells, as readily visualized by staining the underlying actin network with fluorescently labeled phalloidin (van IJzendoorn *et al.*, 1997). As shown in Figure 2A, HepG2 cells acquire PM polarity in time, as evidenced by the ratio BC/100 cells, and reach optimal polarity 3 d after plating (Figure 2A; c.f. van IJzendoorn and Hoekstra, 2000). Treatment of the cells with dibutylryl (db) cAMP or the interleukin-6 family cytokine oncostatin M stimulates polarity development, i.e., apical PM biogenesis in HepG2 cells (Figure 2A, black bars and gray bars, respectively, c.f. van der Wouden *et al.*, 2002), as evidenced by a 2.4- and 1.5-fold increase in the ratio BC/100 cells at 24 and 48–72 h, respectively, after plating. Similar results were obtained with other stable cAMP analogs, and the adenylate

Figure 2. Cell polarity development is positively correlated to $p27^{Kip1}$ expression and G_1 phase arrest. (A) The ratio BC/100 cells was determined at different times in cells cultured on coverslips in normal culture medium (white bars) or normal culture medium supplemented with OSM (gray bars) or dbcAMP (black bars). (B) Cells were cultured in medium supplemented or not with OSM or dbcAMP for different times. Cells were then lysed, and $p27^{Kip1}$ expression was examined by Western blot. A representative blot of three independent experiments is shown. (C) Expression levels of $p27^{Kip1}$ (as depicted in B) were quantified using Scion Image software. The percentage of increase compared with nontreated cells (set to 100%) of three independent experiments is shown ($p < 0.05$ in Student's t test). (D) Cells were cultured in black bars for different times. Twenty-four hours before analysis, BrdU was included in the culture medium. Cells were fixed and stained with anti-BrdU antibodies and the DNA stain Hoechst for immunofluorescence analysis. Note the decreased incorporation of BrdU in OSM- and dbcAMP-treated cells compared with control cells ($p < 0.05$ in Student's t test), indicative for inhibited G_1 -S-phase transition.



cyclase agonist forskolin, but not with sodium butyrate (data not shown; c.f. Zegers and Hoekstra, 1997).

Although the process of cell differentiation generally seems negatively correlated with cellular proliferation, it is not clear whether cell polarity and proliferation are directly, e.g., genetically, linked or that one is an indirect by-effect of the other. We observed that after plating, the expression of p27^{Kip1} increases significantly (approximately twofold) during the first 48 h, after which it remains at a constant level (Figure 2, B and C, white bars). p27^{Kip1} plays a critical role in mediating cell cycle exit by inhibiting the activity of cyclinE/cdk2, a kinase that is required for the transition of cells from the G₁ phase, i.e., the gap between mitosis and the next round of DNA synthesis, into S phase. In agreement with the observed increase in p27^{Kip1} expression, the number of cells that enter S phase and start DNA synthesis is reduced, as evidenced by a decreased incorporation of the thymidine analog BrdU (Figure 2D). Treatment of the cells with oncostatin M (10 ng/ml) enhances the increase in p27^{Kip1} expression during the first 48 h (Figure 2, B and C, gray bars) and results in a more pronounced arrest in the G₁ phase (Figure 2D, gray bars). Similar results were obtained with dibutyryl cAMP (Figure 2, B–D, black bars), well-known to stimulate cell polarity development (Figure 2A, black bars; c.f. Zegers *et al.*, 1997; van IJzendoorn and Hoekstra, 1999, 2000), and after serum deprivation (Figure 3). Together, oncostatin M- and cAMP-stimulated cell polarity, i.e., apical PM biogenesis, is positively correlated with p27^{Kip1} expression and G₁-phase arrest.

Down-Regulation of p27^{Kip1} Expression and Subsequent S-Phase Entry Inhibits Oncostatin M-stimulated Polarity Development

We next investigated whether control of G₁-S-phase progression by p27^{Kip1} is functionally coupled to apical PM biogenesis. To this end, cells were cultured for 36 h in normal cell culture medium to allow expansion of the cells, followed by serum deprivation for another 36 h to synchronize the cells in the G₁ phase. Cells were then stimulated with medium supplemented with (SS) or without (SD) 10% FCS and oncostatin M, in the presence of BrdU for 4 h (Figure 3A). In parallel, cells were grown in normal culture medium for 76 h without medium refreshment for comparison (control; C). As shown in Figure 3, B and C, serum deprivation resulted in increased levels of p27^{Kip1} (~1.4-fold), whereas the subsequent stimulation with serum, but not buffer, resulted in the marked down-regulation of p27^{Kip1} expression (~40%). In agreement with this, few cells entered S phase in serum-deprived cells, whereas, by contrast, ~70% of the cells (~3.5-fold increase) entered S phase after serum stimulation, as evidenced by a massive BrdU incorporation (Figure 3D). The mitotic index in serum-stimulated cell cultures was <3% (unpublished data). Importantly, whereas oncostatin M effectively stimulated cell polarity development in serum-deprived cells by ~45%, as evidenced by an increased ratio of apical PM domains per number of cells, serum-stimulated cells were found to be completely insensitive to oncostatin M stimulation with respect to apical PM biogenesis (Figure 3E).

Similar effects were observed with hydroxyurea (CH₄N₂O₂). Hydroxyurea inactivates ribonucleoside reductase, thereby blocking deoxynucleotide synthesis and arresting the cells in S phase (Balczon *et al.*, 1995; Khodjakov *et al.*, 2002). HepG2 cells were cultured for 36 h in normal culture medium to allow expansion of the cells and then treated with 4 mM hydroxyurea for 36 h, followed by incubation in medium containing OSM and hydroxyurea for 4 h (Figure 4A). Hydroxyurea

prevented DNA synthesis in HepG2 cells as evidenced by reduced BrdU incorporation compared with nontreated cells (73% reduction; c.f. Chouteau *et al.*, 2001). As shown in Figure 4B, hydroxyurea rendered the cells insensitive to the polarity-stimulating effects of OSM very similar as observed in serum-stimulated cells (c.f. Figure 3D).

As an alternative approach to drive cells from G₁ into the S phase, the cells were treated with the mycotoxin fumonisin B1 (FB1) which, in HepG2 cells, results in a decrease in p27^{Kip1} expression of ~75% (Figure 5A). As a consequence, cells treated with FB1 shifted from the G₁ to S phase, evidenced by an increase in the number of cells that had incorporated BrdU (Figure 5A, inset). Treatment of the cells with FB1 inhibits polarity development (Figure 5B). We then examined the effect of FB1-mediated down-regulation of p27^{Kip1} expression on the ability of oncostatin M to stimulate apical PM biogenesis. Oncostatin M failed to stimulate polarity development in FB1-treated cells (Figure 5B), similarly as observed in serum-stimulated or hydroxyurea-treated cells (Figures 3D and 4).

Down-regulation of p27^{Kip1} expression and subsequent G₁-S-phase transition is typically mediated by the increased activity of cyclin-dependent kinase (cdk)2. Indeed, when serum-deprived cells were incubated with the cdk2 inhibitor roscovitine during subsequent serum or FB1 stimulation, cells failed to enter S phase, as evidenced by a lack of increased BrdU incorporation (unpublished data). Importantly, cdk2-inhibited cells retained their sensitivity to the polarity-stimulating effects of oncostatin M after serum or FB1 stimulation (Figure 5B). These data thus suggest that p27^{Kip1} down-regulation by itself is not sufficient but requires cdk2 activity to render the cells insensitive to the polarity-stimulating effects of oncostatin M. Conversely, the down-regulation of cdk2 activity, mediated by OSM-stimulated p27^{Kip1} expression, thus seems an important parameter in OSM-stimulated apical PM biogenesis.

G₁-S Phase Transition Perturbs Oncostatin M-regulated Polarized Membrane Trafficking

In hepatocytes, the SAC, the equivalent of the common endosome in other epithelial cells (van IJzendoorn and Hoekstra, 1999b; van IJzendoorn *et al.*, 2000) plays a crucial role in polarity development. In previous studies, we have demonstrated that polarity development in HepG2 cells requires the activation of a vesicular trafficking route exiting SAC and directed toward the developing BC, which can be monitored by the flow of the fluorescent probes C₆-NBD-SM and C₆-NBD-GalCer (van IJzendoorn and Hoekstra, 1999a,b, 2000). Oncostatin M activates this pathway to stimulate apical PM biogenesis (van der Wouden *et al.*, 2002). The question thus arises whether up-regulation of p27^{Kip1} expression and, consequently, inhibition of G₁-S-phase transition, is required for oncostatin M to exert its effects on membrane traffic. To investigate this, cells were synchronized using serum deprivation/stimulation, according to the incubation scheme depicted in Figure 3A, and SAC was preloaded with C₆-NBD-GalCer as described in detail in *Materials and Methods* (c.f. van der Wouden *et al.*, 2002). A scheme depicting the incubation steps is shown in Figure 1. Note that the trafficking steps necessary for loading SAC, i.e., basolateral endocytosis, apical delivery and apical PM-to-SAC transport, were not visibly affected by the synchronization procedure (Figure 6, A and D). After loading of the fluorescent lipid analogue in SAC, cells were treated with oncostatin M or medium at 4°C (Figure 1, step 5), warmed to 37°C, and lipid flow from SAC to either apical (BC) or basolateral membrane was followed for 20 min in serum-free medium or normal

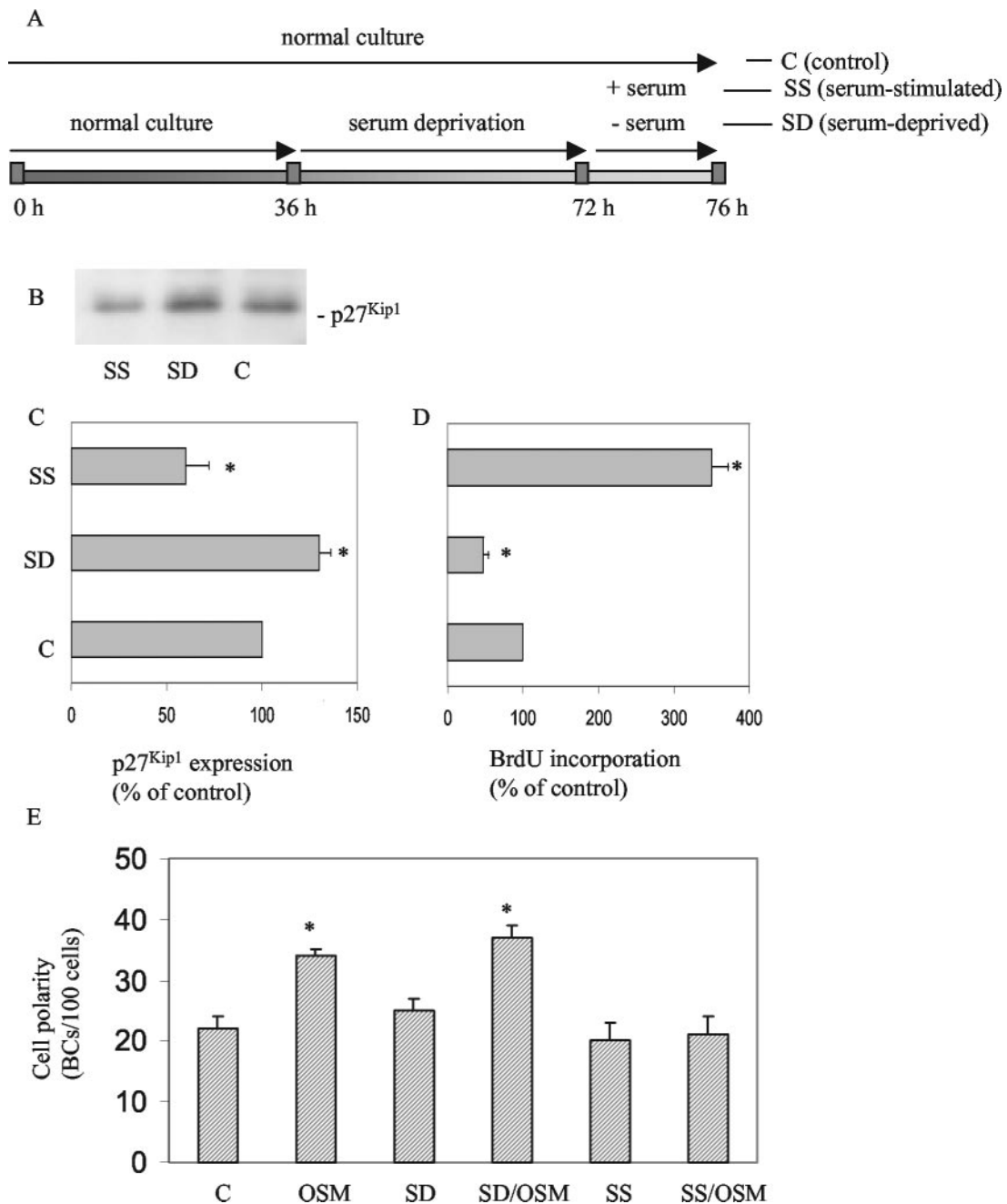


Figure 3. Effect of OSM on polarity development in G₁- and S-phase HepG2 cells. (A) Scheme depicting how cells are synchronized and treated with OSM in the G₁ and S phase of the cell cycle. (B) Expression levels of p27^{Kip1} in serum-deprived (SD), serum-stimulated (SS), and control (C) cells. (C) Quantification of the relative expression levels of p27^{Kip1} in SD, SS compared with C cells. (D) Relative changes in the incorporation of BrdU in SD, SS compared with C cells. (E) Effect of OSM on polarity development in SD, SS, and C cells.

culture medium (Figure 1, step 6). In control (i.e., serum-deprived; G₁) cells, C₆-NBD-GalCer disappeared during this time interval from the BCP, which comprises SAC (where the lipid is located at t = 0; Figure 6A) and BC. Indeed, the percentage of BCP that contained C₆-NBD-GalCer decreased in time (Table 1). The relative distribution of the lipid analogue in the remaining, faintly labeled C₆-NBD-GalCer-positive BCP shows that the probe predominantly resided in SAC and little, if any, movement from SAC to BC could be detected (Table 1; Figure 6B, compare with 6A). Rather, these data indicate that the probe was transported from SAC

to the basolateral domain of the cells, consistent with previous observations (van IJzendoorn and Hoekstra, 1999a, 2000). By contrast, in oncostatin M-treated G₁ cells, C₆-NBD-GalCer remained associated with the BCP region during the 20-min chase, as evidenced by the unchanged percentage of BCP that contained C₆-NBD-GalCer (Table 1). Analysis of the corresponding relative distribution of the probe in the BCP reveals a flow of the lipid analogue from SAC to BC (Table 1; Figure 6C, compare to 6B; cf. van IJzendoorn and Hoekstra, 1999a, 2000). In striking contrast, oncostatin M failed to target C₆-NBD-GalCer from SAC to BC in serum-

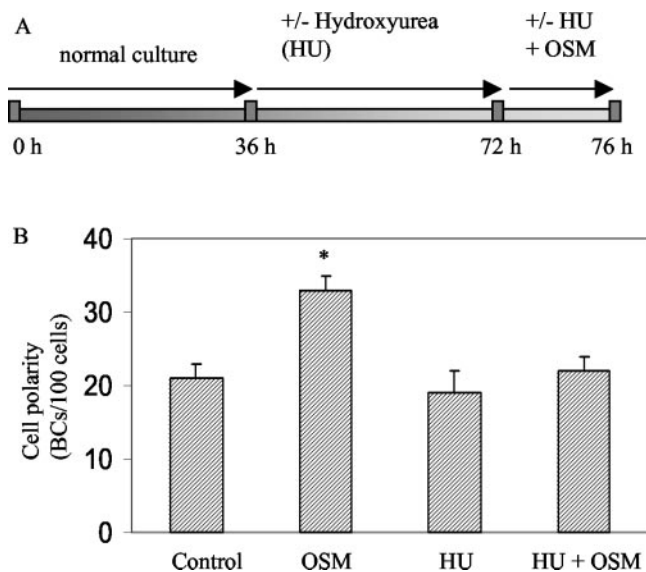


Figure 4. Effect of hydroxyurea (HU) on OSM-stimulated polarity development. (A) Scheme depicting how cells are synchronized with HU (4 mM). (B) HU inhibits the cell polarity-stimulating effects of OSM.

stimulated cells, i.e., cells that had entered S phase (Table 1; Figure 6F, compare with 6E). Thus, in serum-stimulated cells, C₆-NBD-GalCer remained associated with the BCP during the 20-min chase (Table 1), and analysis of the relative distribution of the lipid analogue in the labeled BCP revealed that most C₆-NBD-GalCer localized to SAC (Table 1; Figure 6F), indicating entrapment of the lipid in SAC. Serum stimulation did not affect transport of the fluorescent lipid exiting SAC in the basolateral direction (Figure 6, E vs. B). These data strongly suggest that a minimal level of p27^{Kip1} expression, sufficient for G₁ arrest, is a prerequisite for oncostatin M signaling to specifically activate SAC-to-BC trafficking route and, in this way, to stimulate polarity development.

Treatment of the cells with the cdk2 inhibitor roscovitine shortly before and during serum stimulation, thereby precluding G₁-S phase transition, rescued the ability of OSM to activate SAC-to-BC route followed by C₆-NBD-GalCer (Table 1; Figure 6G, compare with 6C and 6F) and, presumably in this way, apical PM biogenesis (Figure 5), implicating the involvement of cdk2 activity.

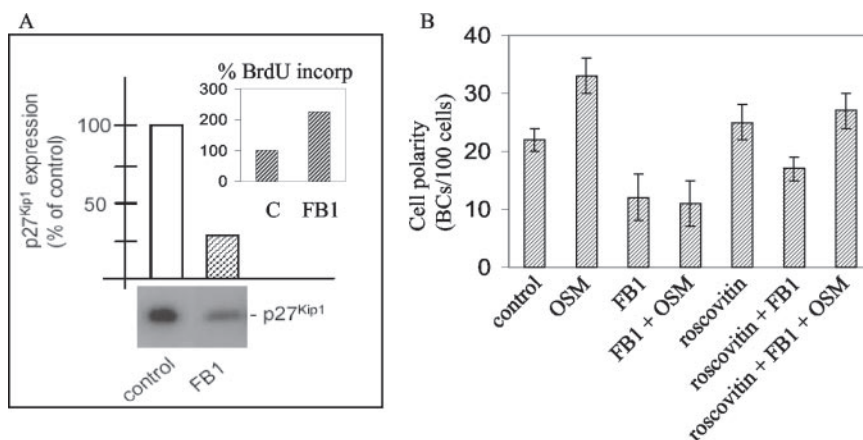


Figure 5. Effect of FB1 on cell cycle and OSM-mediated polarity development. (A) FB1 down-regulates p27^{Kip1} expression (blot and quantification) and promotes S-phase entry, as evidenced by BrdU incorporation (inset). (B) FB1 inhibits OSM-stimulated polarity development in HepG2 cells. Note that the cdk2 inhibitor roscovitine restores the stimulatory effect of OSM on polarity development in FB1-treated cells.

G₁-S-Phase Transition Inhibits OSM-stimulated Recruitment of PKA to the Centrosomal Region

The ability of OSM to activate SAC-to-apical surface route requires cAMP/PKA activity, as evidenced by using pharmacological inhibitors of PKA (van der Wouden *et al.*, 2002). Although OSM did not give rise to increased cAMP or PKA expression levels, a significant recruitment of PKA to the centrosome region was noted (van der Wouden *et al.*, 2002). To determine whether the OSM-stimulated recruitment of PKA to the centrosome region corresponds to its ability to activate the SAC-to-BC pathway, we examined the effect of G₁-S-phase transition on the ability of OSM to stimulate centrosomal PKA recruitment. For this, cells were synchronized in G₁ by serum deprivation and subsequently incubated for 4 h in serum-free medium (which maintains the cells in G₁) or in FCS-containing medium (which drives the cells into the S-phase), both supplemented with OSM. Cells were then fixed and stained with antibodies raised against the centrosomal protein γ -tubulin and PKA. The percentage of PKA-positive centrosomes (identified by γ -tubulin) was then determined. As shown in Table 2, OSM stimulated the recruitment of PKA to centrosome region in G₁ cells (~24% more γ -tubulin-labeled centrosomes were positive for PKA). Illustrative immunofluorescence pictures are shown in Figure 7, A–F. In striking contrast, OSM failed to recruit PKA to the centrosome region in S-phase cells (Table 2). In fact, 13% less γ -tubulin-labeled centrosomes in serum-stimulated cells displayed PKA association, and OSM failed to stimulate PKA recruitment at the centrosomal region (Table 2 and Figure 7, G–L). In serum-stimulated cells, but not in serum-deprived cells, some PKA localized to the nucleus. Given that PKA is required for OSM to activate the SAC-to-BC transport pathway and apical PM biogenesis, the apparent inability of OSM to recruit PKA to the centrosome region upon G₁-S-phase transition possibly is part of the underlying mechanism responsible for rendering the cells insensitive to OSM with regard to apical PM biogenesis.

DISCUSSION

In this study, we have addressed the functional relationship between cell cycle regulation and the acquisition of a polarized phenotype, i.e., apical PM biogenesis, in hepatic HepG2 cells in response to OSM signaling. First, we demonstrate that (OSM-stimulated) apical PM biogenesis in HepG2 cells displays a strong positive correlation with expression levels of the cell cycle regulatory protein p27^{Kip1} and subsequent G₁-phase arrest. Indeed, exposure of the cells to OSM en-

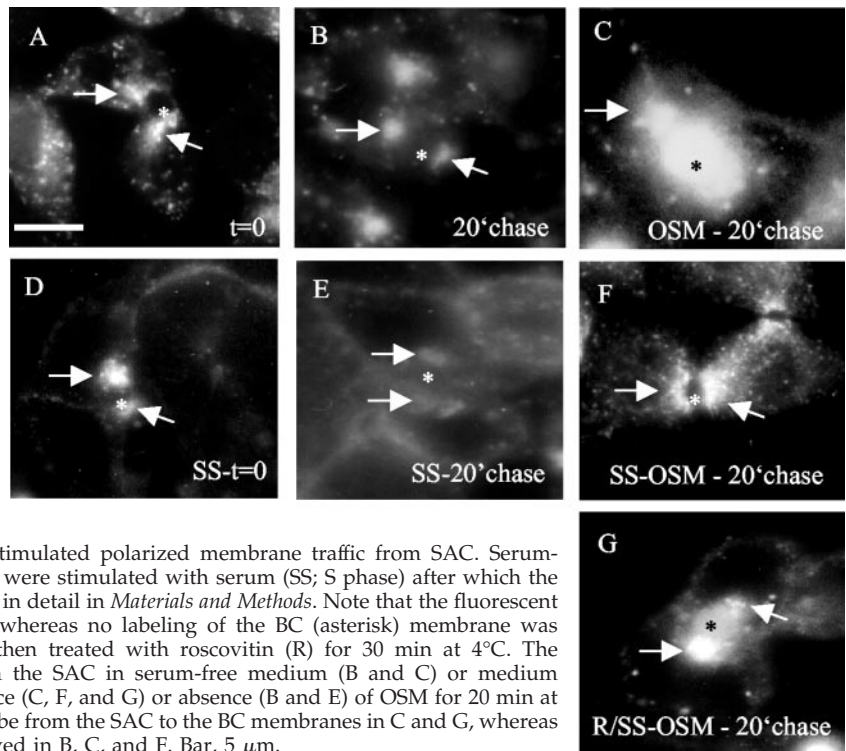


Figure 6. G₁-S-phase transition perturbs OSM-stimulated polarized membrane traffic from SAC. Serum-deprived cells kept in serum-free medium (G₁) or were stimulated with serum (SS; S phase) after which the SAC was loaded with C₆NBD-GalCer as described in detail in *Materials and Methods*. Note that the fluorescent probe predominantly labeled the SAC (arrows), whereas no labeling of the BC (asterisk) membrane was observed. In some experiments (G), cells were then treated with roscovitin (R) for 30 min at 4°C. The fluorescent probe was subsequently chased from the SAC in serum-free medium (B and C) or medium supplemented with serum (SS, E–G) in the presence (C, F, and G) or absence (B and E) of OSM for 20 min at 37°C. Note the redistribution of the fluorescent probe from the SAC to the BC membranes in C and G, whereas no redistribution from SAC to BC could be observed in B, C, and F. Bar, 5 μ m.

hances the expression of p27^{Kip1} and prevents entry of the cells in S phase as evidenced by the inhibition of BrdU incorporation (Figure 2). This is in agreement with the findings of Klausen *et al.* (2000), which showed that OSM inhibits cell cycle progression by preventing p27^{Kip1} degradation in HepG2 cells. Importantly, our data indicate that apical PM biogenesis stimulated by OSM is critically dependent on the ability of OSM to control p27^{Kip1}/cdk2-mediated G₁-S-phase progression. Thus, asynchronous cells readily respond to OSM signaling with regard to apical PM biogenesis, and serum deprivation, which results in increased p27^{Kip1} expression and synchronization of the cells in G₁-phase, enhances the stimulatory effect of OSM (Figure 3). In striking contrast, serum- or FB1-stimulated down-regulation of p27^{Kip1} expression levels, allowing G₁-S-phase transition to occur, effectively renders the cells insensitive to the cell polarity-stimulating effects of OSM (Figures 2 and 3). Indeed, cells similarly acquire insensitivity to the polarity-stimulating effects of OSM after hydroxyurea-induced S-phase arrest (Figure 4). p27^{Kip1} down-regulation results in an expected net increase in cdk2 activity, which plays an important role in G₁-S-phase transition (Ekholm and Reed,

2000; Woo and Poon, 2003). Inhibition of cdk2 activity in HepG2 cells with roscovitin prevented G₁-S-phase transition and kept the cells responsive to the polarity-stimulating effects of OSM, even in the serum- or FB1-treated cells. Together, our data indicate that extracellular signal- and p27^{Kip1}/cdk2-regulated cell cycle progression controls de novo apicalization of the cell surface.

Serum-stimulated G₁-S-phase transition effectively inhibits the OSM-stimulated and PKA-dependent activation of a membrane transport pathway exiting the SAC and directed toward the developing apical surface (Table 1 and Figure 6). The activation of this membrane trafficking pathway has previously been demonstrated to play an important role in apical-basolateral polarity development in HepG2 cells (van Ijzendoorn and Hoekstra, 1999a,b; van Ijzendoorn *et al.*, 2000; Hoekstra *et al.*, 2004). OSM-stimulated membrane trafficking from SAC to the developing apical surface is dependent on intact microtubules (unpublished data). Serum-stimulated G₁-S-phase transition, however, did not visibly affect the spatial organization of SAC (Figure 6) and also the distribution of the microtubule cytoskeleton was not visibly altered (unpublished data), supporting the notion that se-

Table 1. G₁-S-phase transition perturbs OSM-stimulated polarized membrane traffic from SAC

	0-min chase	20-min chase G ₁ -control	20-min chase G ₁ -OSM	20-min chase SS-OSM	20-min chase R/SS-OSM
% Positive BCP	89 (2)	63 (4)*	88 (3)	90 (2)	84 (4)
Relative distribution in BCP					
SAC	84 (3)	79 (3)	40 (2)*	89 (3)	30 (4)*
BC + SAC	17 (2)	21 (3)	30 (4)*	11 (2)	20 (1)
BC	3 (1)	2 (1)	35 (3)*	3 (1)	45 (3)*

* p < 0.05.

Table 2. Effect of serum-stimulated G₁-S-phase transition on OSM-stimulated recruitment of PKA to the centrosomal region

	% Increase in PKA-positive centrosomes
Control	0
OSM	+24 ± 4*
SS-control	-13 ± 4*
SS-OSM	-12 ± 3*

* $p < 0.01$ (OSM vs. control; SS-control vs. control; SS-OSM vs. OSM).

SS, serum starved.

rum-stimulated cells had not reached prophase during which microtubules are rearranged to form spindles, and indicating that the observed S-phase entry-induced impairment of OSM-regulated polarized membrane traffic from SAC is not likely the result of a cell cycle-related reorganization of the microtubule cytoskeleton.

The OSM-stimulated and PKA-dependent reorganization of polarized membrane trafficking from SAC coincides with a stimulated recruitment of PKA to the centrosomal region (van der Wouden *et al.*, 2002). The SAC and other organelles involved in polarized sorting, e.g., the Golgi apparatus, are typically concentrated in the centrosomal region (Hobdy-Henderson *et al.*, 2003; Rios and Bornens, 2003), and both the centrosome and associated organelles typically face specialized cell surface domains, e.g., the leading lamella in migrating fibroblasts (Ueda *et al.*, 1997) and the apical PM domain

in epithelial cells (Buendia *et al.*, 1990). Moreover, in differentiated G₁ cells, one of the centrosomes moves to the apical surface to form the basal body of the primary cilium, a highly specialized membrane domain that extends from the apical surface. The functional relevance for the clustering of SAC and Golgi around the centrosome is not clear, but it may serve a purpose in integrating complex signaling cascades (Lange, 2002; Rios and Bornens, 2003). In addition, recent evidence suggests that the centrosome positions organelles in proximity to specific target membranes. This may then facilitate or reinforce cytoskeleton remodeling and/or membrane exchange between organelles and the cell surface necessary for the biogenesis of specialized PM domains, such as the apical surface (Wald *et al.*, 2003) or the furrow during cytokinesis (Riggs *et al.*, 2003). Serum-triggered G₁-S-phase transition effectively inhibits the OSM-stimulated recruitment of PKA to the centrosomal region (Table 2 and Figure 7). This is in agreement with the finding that the displacement of PKA from centrosomes positively modulates G₁-S-phase transition (Felicello *et al.*, 2000). Interestingly, very similar to the observed inhibition of de novo polarity development upon G₁-S-phase transition (Figures 3 and 4), the stimulatory effect of OSM on apical PM biogenesis in HepG2 cells is abolished when cAMP/PKA activity is inhibited (van der Wouden *et al.*, 2002). These data suggest that recruitment of PKA to the centrosomal region is required for OSM to activate a membrane traffic route from SAC to the developing apical surface, and in this way, stimulate apical PM biogenesis. This underscores the importance of the centrosomal region as a specialized site where complex cellular events, including cell cycle progression and membrane traffic, are coordinately integrated (Rieder *et al.*,

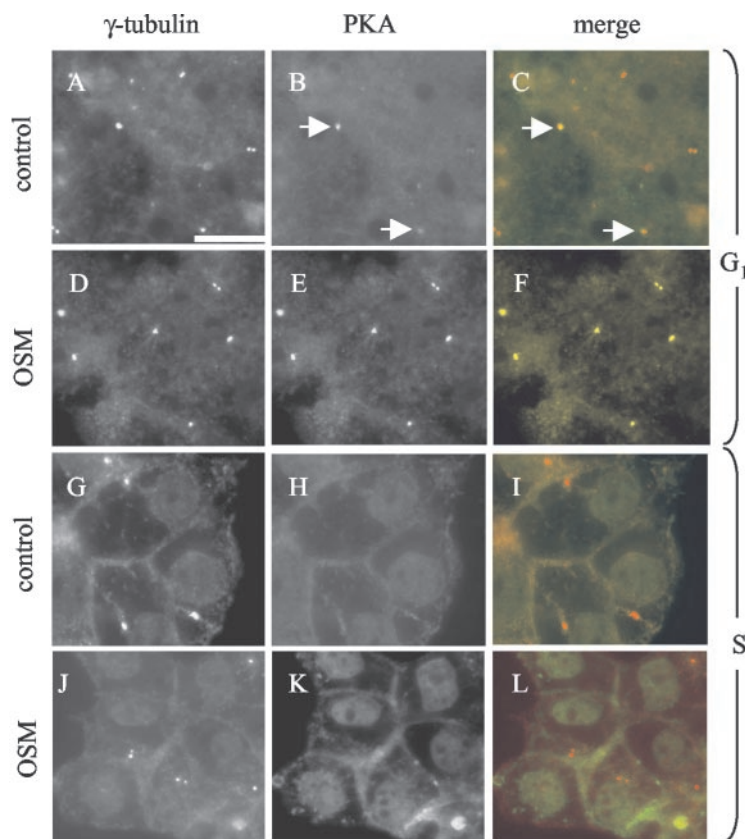


Figure 7. Effect of serum-stimulated G₁-S-phase transition on OSM-stimulated recruitment of PKA to the centrosomal region. Serum-deprived cells were kept in serum-free medium (to keep them in G₁; A–F) or were stimulated with serum (to drive them in the S phase; G–L) in the presence (D–F, J–L) or absence (A–C, G–I) of OSM. The cells were then fixed and labeled with antibodies against γ -tubulin and PKA as described in *Materials and Methods*. The percentage of γ -tubulin-positive centrosomes that were labeled with PKA was determined and representative pictures were taken. Note that OSM stimulates the recruitment of PKA to γ -tubulin-positive centrosomes (dotted appearance) in G₁ cells (compare D–F with A–C) but not in S-phase cells (compare J–L with G–I). Arrows in B and C point to centrosomes positive for both γ -tubulin and PKA. In D–F, all γ -tubulin-positive centrosomes shown are also positive for PKA. C, F, I, and L show merged images with γ -tubulin in red and PKA in green. Bars, 10 μ m.

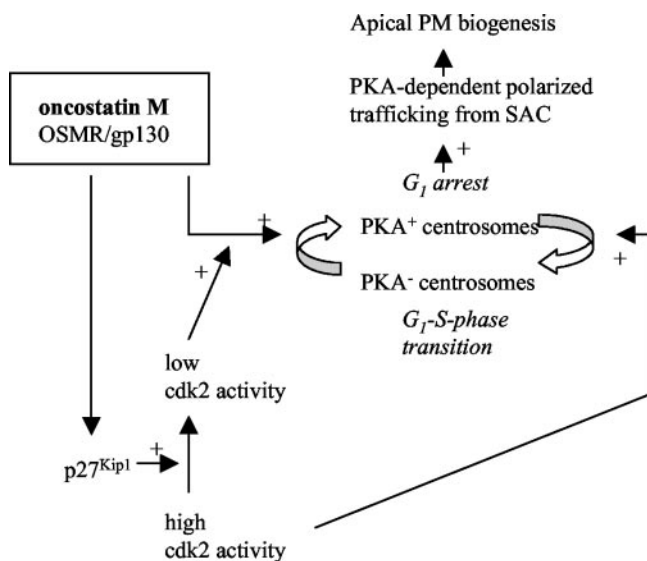


Figure 8. Working model. A detailed explanation is provided in Discussion.

2001), and it implicates OSM as an important regulating factor in this. At present, a molecular link between cdk2 activity at the G_1/S -boundary and PKA association at the centrosome is unclear. However, such a link is not unprecedented because mitotic CDK1 activity has been demonstrated to displace PKA from centrosomal A-kinase anchoring proteins. We are currently investigating the molecular mechanism by which OSM stimulates PKA recruitment to the centrosomal region and the identification of OSM-controlled PKA targets.

Based on the data, we propose a model (Figure 8) in which OSM signaling enhances p27^{Kip1} expression, and consequently down-regulates cdk2 activity, resulting in G_1 arrest. Low cdk2 activity allows OSM-stimulated recruitment of PKA to the centrosomal region that is essential for mediating the OSM-activated membrane exchanges between SAC and the developing apical surface. On G_1 -S-phase transition, induced by circulating growth factors or mitogenic mycotoxins such as FB1 (Schroeder *et al.*, 1994; this study), the increasing cdk2 activity may not be sufficiently down-regulated by OSM and therefore interferes with OSM-mediated recruitment of PKA to the centrosomal region. This then impairs the regulated membrane supply from the pericentrosomal SAC to the cell surface and hence apical PM biogenesis. This model underscores the recently suggested role of endosomal recycling in the spatial-temporal control of plasma membrane insertion that is required for cell morphogenesis (Lecuit, 2003; Pelissier *et al.*, 2003), e.g., the development of the apical domain, and it places PKA-regulated polarized plasma membrane recycling under control of p27^{Kip1}/cdk2-regulated G_1 -S-phase transition.

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